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Probing Metal Binding in the 8–17 DNAzyme by Tb^{III} Luminescence Spectroscopy

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Abstract: Metal-dependent cleavage activities of the 8–17 DNAzyme were found to be inhibited by Tb^{III} ions, and the apparent inhibition constant in the presence of 100 μM of Zn^{II} was measured to be 3.3 ± 0.3 μM. The apparent inhibition constants increased linearly with increasing Zn^{II} concentration, and the inhibition effect could be fully rescued with addition of active metal ions, indicating that Tb^{III} is a competitive inhibitor and that the effect is completely reversible. The sensitized Tb^{III} luminescence at 543 nm was dramatically enhanced when Tb^{III} was added to the DNAzyme–substrate complex. With an inactive DNAzyme in which the GT wobble pair was replaced with a GC Watson–Crick base pair, the luminescence enhancement was slightly decreased. In addition, when the DNAzyme strand was replaced with a complete complementary strand to the substrate, no significant luminescence enhancement was observed. These observations suggest that Tb^{III} may bind to an unpaired region of the DNAzyme, with the GT wobble pair playing a role. Luminescence lifetime measurements in D_2O and H_2O suggested that Tb^{III} bound to DNAzyme is coordinated by 6.7 ± 0.2 water molecules and two or three functional groups from the DNAzyme. Divalent metal ions competed for the Tb^{III} binding site(s) in the order $Co^{II} > Zn^{II} > Mn^{II} > Pb^{II} > Ca^{II} \approx Mg^{II}$. This order closely follows the order of DNAzyme activity, with the exception of Pb^{II}. These results indicate that Pb^{II}, the most active metal ion, competes for Tb^{III} binding differently from other metal ions such as Zn^{II}, suggesting that Pb^{II} may bind to a different site from that for the other metal ions including Zn^{II} and Tb^{III}.

Keywords: cofactors · DNA · inhibitors · luminescence · sensors

Introduction

Metal ions are important cofactors in biology for expanding the functionality of biomolecules. They are found in one third of structurally characterized proteins and almost half of all proteins overall. Ribozymes and deoxyribozymes (or DNAzymes), with even smaller numbers of available functional groups, depend even more on metal ions for their functions than proteins do. Not surprisingly, most ribozymes and deoxyribozymes require metal ions for structure and activity.[1–5] A good example is the 8–17 DNAzyme, isolated by in vitro selection under several different sets of conditions for cleaving substrate DNA possessing an RNA base at the cleavage site.[6–10] It was found to be its most active in the presence of Pb^{II}, with the order of relative activities following: $Pb^{II} \gg Zn^{II} \gg Mn^{II} \approx Co^{II} > Ni^{II} > Mg^{II} \approx Ca^{II} > Sr^{II} \approx Ba^{II}$. The high reactivity and metal ion selectivity of this DNAzyme have allowed its conversion into metal ion sensors.[11–14]

In contrast with the abundance of research into and information about metal-binding sites in proteins, our understanding of metal-binding sites in nucleic acids is lacking. The information is critical to enriching fundamental knowledge about metal–nucleic acid coordination chemistry and its role in the function of ribozymes and deoxyribozymes, and also to application of the knowledge to the design of better sensors. To this end, a number of biochemical and biophysical studies have been carried out on nucleic acids, including ribozymes.[15–30] For metal-binding sites in DNAzymes, even less is known than for studies on ribozymes. Because of the interesting applications of 8–17 DNAzymes as metal ion sensors, it would be interesting to find out how the DNAzyme carries out its reactions in the presence of different metal ions and what structural features are responsible for its selectivity. Recently, FRET studies on the 8–17 DNAzyme both in the bulk solution[31] and at single-molecule level[32] have been carried out. The results strongly

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suggested that metal-dependent folding played an important role in DNAzyme activity in the presence of some metal ions, but not others. Since these FRET studies probe only the metal-dependent global folding, more information is required in order to obtain detailed information about the local metal-binding sites in the DNAzyme.

Many spectroscopic techniques have been shown to be valuable tools to provide such local metal-binding information in proteins, especially when diffraction-quality crystals cannot be obtained. For example, spectroscopic titrations have been used to study metal-binding in ribozymes.[18,20,33] A special challenge for ribozymes and deoxyribozymes, however, is that many active metal ions, such as Mg^{II}, Ca^{II}, Zn^{II}, and Pb^{II}, do not have rich spectroscopic properties such as color and magnetism, making it difficult to probe the metal-binding sites by metal-based spectroscopic techniques. An effective strategy is to substitute those metal ions with spectroscopically rich metal ions that are either active[34–36] or inhibitive competitively.[37–40]

One of the most often used spectroscopic probes is the trivalent lanthanide ion, Ln^{III}, which shows sensitized luminescence by energy transfer from nucleic acids with different structural or sequential sensitivity.[37–45] Lanthanide ions have been shown to be good probes for studying metal-binding sites in proteins,[46–49] tRNA,[37] ribozymes such as hammerhead[38,39] and hairpin ribozymes,[40] and a lead-dependent DNAzyme.[50] Tb^{III} and Eu^{III}, for example, were shown to inhibit the hammerhead ribozyme cleavage reaction by competing for a binding site with Mg^{II} ion, which made it possible to study the Mg^{II} binding sites.[38,39] Tb^{III} also inhibits its hairpin ribozyme activity by competing with all cation cofactors.[40] Lanthanide ions were used as effective cofactors for a Pb^{II}-dependent DNAzyme to probe metal-binding sites,[50] and luminescence lifetime experiments were used to find the number of coordinated waters on the lanthanide ions binding to the hammerhead ribozyme[39] and RNA hairpin loops.[51]

In this study, we report that Tb^{III} is a competitive and reversible inhibitor for the Zn^{II}- and Pb^{II}-dependent 8–17 DNAzyme activity. This finding allowed us to use sensitized Tb^{III} luminescence spectroscopy to provide information about the metal-binding site in the 8–17 DNAzyme, such as possible locations and number of functional groups involved in metal binding in the DNAzyme. Interestingly, we found that Pb^{II}, the most active metal ion, competes for Tb^{III} binding differently from other metal ions such as Zn^{II}, suggesting that Pb^{II} may bind to a site different from that involved with the other metal ions including Zn^{II} and Tb^{III}.

Results

DNA constructs: The sequences of the enzyme strand (called 17E) and substrate strand (called 17S) of the 8–17 DNAzyme are shown in Figure 1A. Previous studies had shown that a single base change—T2.1C—producing a GC base pair instead of the GT wobble pair adjacent to the cleavage site abolished the metal-dependent activity.[6,8,52] Therefore this variant, called 17E(T2.1C)-S (Figure 1B), was used as a control for inactive DNAzyme–substrate complex in this study. A further control is a complete complementary double-stranded DNA, called 17S duplex (Figure 1C), in which the enzyme strand has been replaced by a completely complementary strand (Anti-17S) to the substrate to form double-stranded DNA. For the luminescence spectroscopic studies involving divalent metal ions as an active cofactor, a non-cleavable substrate (17DDS) in which the ribonucleotide at the cleavage site has been replaced by a deoxyribonucleotide (Figure 1D) was used in order to prevent cleavage of the substrate strand.

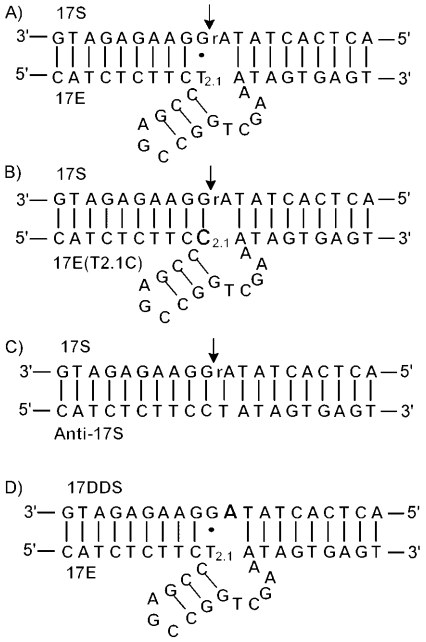

Figure 1. Sequences and secondary structures of: A) 8–17 DNAzyme consisting of the 17E enzyme strand and the 17S substrate strand, B) an inactive mutant complex (17E(T2.1C)-S) containing a GC base pair instead of the GT wobble pair immediately adjacent to the cleavage site, C) 17S duplex, a complete complementary double-stranded DNA with an anti-17S instead of a 17E enzyme strand, and D) non-cleavable complex (17E-DDS), in which the ribonucleotide (rA) at the cleavage site has been replaced with a deoxyribonucleotide. The arrow indicates the cleavage site.

Inhibition of the 8–17 DNAzyme activity by Tb^{III}: As reported previously,[8,52] the 8–17 DNAzyme is active in the presence of Zn^{II} (100 μM), showing a fast increase in cleavage product with increase in time (Figure 2). When Tb^{III} was added to the above reaction mixture to a final concentration of 20 μM at the 5 min time point, however, no further reaction product was observed and the reaction was stopped immediately (Figure 2). This single-chase experiment indicates that Tb^{III} is an effective inhibitor of the reaction. In a double-chasing experiment, Zn^{II} (2 mM final concentration) was added 15 min after Tb^{III} had been added to the reaction mixture. Upon addition of the Zn^{II}, an immediate increase

in the reaction product was again observed, and the final percentage product is almost identical to that of Tb^{III}-free reaction, suggesting that the Tb^{III} inhibitory effect is fully reversible and that Tb^{III} ions do not permanently damage the active structure of the DNAzyme–substrate complex.

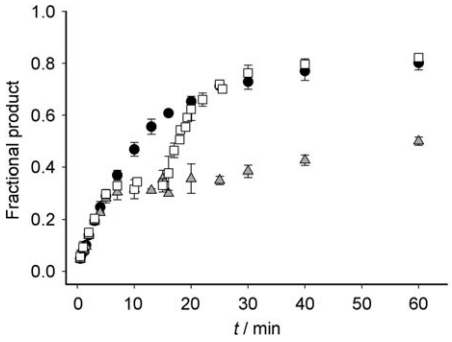

Figure 2. Effect of Tb^{III} on Zn^{II}-dependent substrate cleavage catalyzed by 17E. The reaction was carried out with 17S (1 nM) and 17E (5 μM) in MES buffer (50 mM, pH 6.0). The reaction was initiated with Zn^{II} (100 μM, ●). In the single-chased reaction (△), $TbCl_3$ (20 μM) was added to the reaction mixture 5 min after addition of Zn^{II}. In the double-chased reaction (□), Zn^{II} (2 mM) was also added 15 minutes after addition of $TbCl_3$.

To obtain the apparent Tb^{III} inhibition constant—$K_{i\,app}$—the extent of inhibition of Zn^{II}-dependent DNAzyme activity was plotted against increasing Tb^{III} concentrations (Figure 3A). A spectral fitting to a competitive inhibition equation as described in the Experimental Section resulted in a $K_{i\,app}$ value of 3.3 ± 0.3 μM Tb^{III} in the presence of Zn^{II} (100 μM), indicating a much higher affinity of Tb^{III} towards the DNAzyme than that of Zn^{II}. The apparent inhibition constants increased linearly as concentration of Zn^{II} increased, as shown in Figure 3C, suggesting that Tb^{III} is a competitive inhibitor to Zn^{II}. To find out whether nonspecific binding of Tb^{III} ions could be captured, we fit the plots with a modified equation that includes a second, nonspecific Tb^{III} binding term: $k=k_{max}[S]/[K_s\{1+[I](1/K_{i\,app1}+1/K_{i\,app2})\}+[S]]$. Such a fitting using a second term did not result in improvement in the curve fitting, with the same R^2 values for all fitting. Moreover, the errors in the inhibition constants increased significantly, indicating that fitting the plot with a single binding site provides more reliable values.

Tb^{III} was also observed to inhibit the Pb^{II}-dependent cleavage reaction catalyzed by the DNAzyme in a similar way as in the Zn^{II}-dependent reaction (data not shown). In agreement with the fact that Pb^{II} is more active than Zn^{II},[8,52] Pb^{II} (100 μM) was enough to recover the full activity inhibited by Tb^{III} (20 μM). As shown in Zn^{II} case, the observed rate constant (k_{obs}) of the Pb^{II}-dependent reaction was observed to decrease as Tb^{III} concentration increased while final cleavage percentage remained constant, independent of Tb^{III} concentration (Figure 3B). It was difficult to obtain a Tb^{III} inhibition constant for the Pb^{II}-dependent reaction, however, because the apparent binding constant of

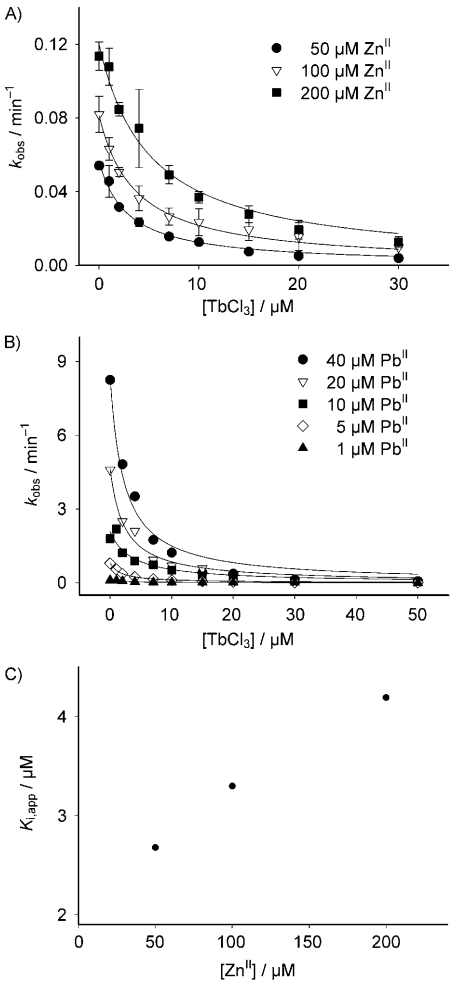

Figure 3. A) Extent of inhibition of the 8–17 DNAzyme activity as a function of Tb^{III} concentration in the presence of Zn^{II}; k is the observed rate constant (k_{obs}) measured from activity assays as described in the Experimental Section. The data were fitted to a competitive inhibition equation as described in the Experimental Section. The reaction was carried out with 17S (1 nM) and 17E (5 μM) in the presence of Zn^{II} and various concentrations of Tb^{III} in MES (50 mM, pH 6.0). B) Same as a), but in the presence of Pb^{II}. C) Apparent inhibition constants—$K_{i\,app}$—obtained from a) in the presence of various concentrations of Zn^{II}.

Pb^{II} (13.5 μM) is too strong to allow study of competitive inhibition at suitable conditions. These results, even though not quantitative, indicate that Tb^{III} is also a competitive inhibitor of Pb^{II}.

Terbium luminescence spectroscopy: Since the active Zn^{II} and Pb^{II} metal ions have a limited spectroscopic signature, it has been difficult to obtain structural information about the active metal-binding site(s) of the 8–17 DNAzyme. The strong reversible Tb^{III} inhibition of Zn^{II}- and Pb^{II}-dependent DNAzyme activity provides an opportunity to probe at or close to the active binding site by Tb^{III} luminescence spectroscopy. In order to avoid cleavage of the substrate strand during the spectroscopic studies involving divalent metal ions, a noncleavable substrate (17DDS) in which the ribonucleotide at the cleavage site had been replaced by a deoxyri-

bonucleotide (Figure 1D) was used for measuring luminescence. In the absence of Tb^{III} the DNAzyme did not show luminescence in the 450–650 nm region (Figure 4). Addition of Tb^{III} to a final concentration of 10 μM resulted in four strong luminescence peaks (488, 543, 584, and 620 nm), typical of the fine structure of a Tb^{III} ion. Solvated Tb^{III} ion alone at 100 μM showed very weak luminescence from direct excitation of the Tb^{III}. These results indicated that Tb^{III} was bound to the DNAzyme and that the observed luminescence was due to the effective energy transfer from the DNAzyme to the Tb^{III} ion. The same results were obtained with cleavable substrate complex (17E-S) in the absence of Zn^{II}. This result is consistent with Tb^{III} effectively inhibiting the DNAzyme cleavage reaction through binding to the DNAzyme.

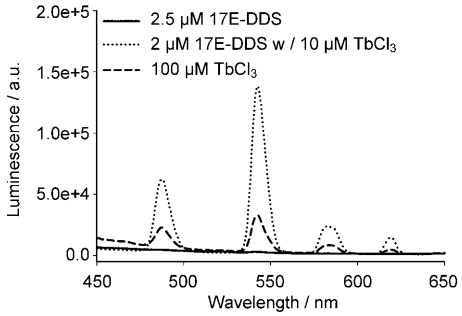

Figure 4. Sensitized Tb^{III} luminescence spectra in the presence and the absence of 17E-DDS in MES (50 mM, pH 6.0) and $ZnCl_2$ (100 μM).

To obtain more quantitative information on Tb^{III} binding to the DNAzyme, the active DNAzyme–substrate complex (17E-S) in MES (50 mM, pH 6.0) and $NaNO_3$ (50 mM) was titrated with Tb^{III} ion, and the corresponding sensitized luminescence at 543 nm was measured as shown in Figure 5. Gradual luminescence enhancement was observed as Tb^{III} concentrations increased from 0 to 20 μM, at which Tb^{III} inhibits DNAzyme activity. Interestingly, the inactive variant 17E(T2.1C)-S complex showed the same trend as in the case of 17E-S, but the luminescence intensity was always lower than the corresponding intensity from active complex. Three different experimental sets on two different dates showed consistent results relating to the slight difference in the luminescence between the 17E-S and the inactive variant. Furthermore, the 17S–anti17S duplex containing the same 17S sequence, but with the completely complementary sequence in the other strand (Figure 1C) showed little enhancement of the luminescence by Tb^{III}. This result is consistent with the observation that Tb^{III} ions bound to double-stranded DNA do not enhance luminescence.[41,42] Therefore, the clear enhancement of the luminescence with the DNAzyme indicates that Tb^{III} ion(s) bind(s) to the unpaired bases at the bulge and the stem loop around the active core region in the enzyme strand. The lower luminescence with the inactive variant suggests that Tb^{III} binding may involve the GT wobble pair, which is critical to the cleavage activity.

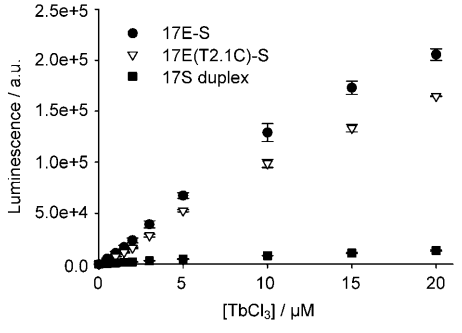

Figure 5. Sensitized Tb^{III}-dependent luminescence at 543 nm as a function of Tb^{III} concentration. Concentrated $TbCl_3$ solutions were titrated into DNA solutions (2 μM, 500 μL) in MES (50 mM, pH 6.0) and $NaNO_3$ (50 mM); 17E-S is the wild-type DNAzyme with a cleavable substrate, 17E(T2.1C)-S is an inactive DNAzyme with a cleavable substrate, and 17S duplex is a completely base-paired duplex.

The luminescence spectra cannot be fit to a standard binding equation as the curves do not reach saturation at high metal ion concentrations, indicating that the binding constant is >10 μM. The difference between the binding constant (>10 μM, Figure 5) and the apparent inhibition constant ($K_{i\,app}=3.3$ μM; Figure 3) implies the presence of more than one single Tb^{III} binding site in the DNAzyme, with different binding affinities, and that one of the binding sites is critical for the inhibition. Also, when the titration was extended above 20 μM, the concentration used in the activity assay shown in Figure 2, the luminescence intensity increased dramatically and finally saturated at around 40 μM Tb^{III} (data not shown). This observation is most likely the result of the disruption of the secondary structure of the DNAzyme resulting in nonspecific binding of Tb^{III} in the region of high Tb^{III} concentrations. It has been shown that Tb^{III} binding to bases can induce conformational changes in DNA by interrupting hydrogen bonding between base pairs.[53] Simultaneous chelation of the phosphate group and the N-7 guanine by Tb^{III} alters the geometry of the sugar–phosphate backbone and the stacking interaction between the bases in double-stranded DNA. However, it is still possible that Tb^{III} ions may cooperatively bind to other site(s) with weak affinities in the catalytic core area containing unpaired base pairs after saturation of the site(s) with strong affinities.

Luminescence lifetime measurements: Another advantage of lanthanide luminescence is that its lifetime measurement in aqueous solution can provide information relating to the inner-sphere coordination environment of the ion.[39,54–56] The primary pathway for nonradiative decay of the lanthanide ion excited state is through the O–H vibrational manifold of bound water molecules. The decay rate of this process is directly proportional to the number of OH oscillators in the first coordination sphere. Moreover, this nonradiative relaxation path exhibits a very large isotope effect such that O–D oscillators are much less efficient at affecting this relaxation process. The difference in the luminescence decay rate in H_2O versus D_2O has been shown empirically to vary

linearly with the number of water molecules (q) in the inner coordination sphere:

$$q = A\,(\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1}) \quad (1)$$

where $\tau_{D_2O}^{-1}$ is the luminescence lifetime in D_2O and $\tau_{H_2O}^{-1}$ is the lifetime in H_2O. The constant A is 4.2 for Tb^{III} for τ^{-1} values in ms^{-1}.[57]

To measure the luminescence lifetime of Tb^{III}, the luminescence decays in D_2O and in H_2O were measured, and their normalized plots are shown in Figure 6. The luminescence decay curves were fit to a single exponential decay equation with $R^2 > 0.999$, suggesting a single class of Tb^{III} binding to the DNAzyme. As expected, the lifetime of Tb^{III}–DNAzyme adduct in H_2O ($\tau^{-1} = 1.97\ ms^{-1}$) was shorter than that in D_2O ($\tau^{-1} = 0.368\ ms^{-1}$), due to the efficient vibrational energy transfer to H_2O bound to Tb^{III}. The number of water molecules coordinated to Tb^{III} in the enzyme–substrate complex is estimated to be 6.7 ± 0.2 according to Equation (1). Since it has been found that nine water molecules are coordinated to Tb^{III}, two or three water molecules are thus replaced by functional groups in the DNAzyme upon binding.

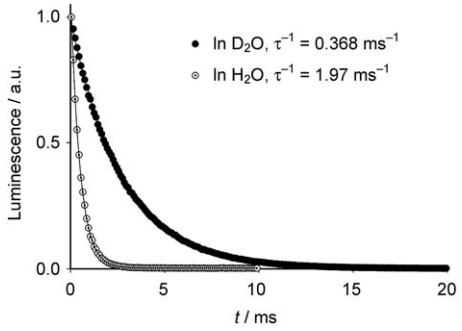

Figure 6. Normalized Tb^{III} luminescence decay plots in D_2O and in H_2O for lifetime measurement. Lifetimes (τ) were obtained by fitting the plots to single exponential decay curves (solid lines). An aliquot of 17E-S (2 μM) was preincubated with $TbCl_3$ (15 μM) in MES (50 mM, pH/pD 6.0) and $NaNO_3$ (50 mM).

Metal competition studies monitored by Tb^{III} luminescence spectroscopy: To provide further support for the conjecture that Tb^{III} binds at or close to the active metal-binding site(s), sensitized Tb^{III} luminescence intensity at 543 nm was monitored after different metal ions had been titrated into the preequilibrated Tb^{III}–DNAzyme adducts. The DNAzyme construct with the noncleavable substrate (17DDS) (Figure 1D) was used in order to avoid cleavage of the substrate strand during the luminescence measurements. Use of different counter anions of the same metal cation, such as $CoCl_2$ and $Co(NO_3)_2$, or $MnCl_2$ and $Mn(OAc)_2$, made little difference in luminescence intensity (data not shown). This result is consistent with the findings that DNAzyme activity and sensor ability are independent of anions present in the solution. Typical metal competition curves for several metal ions are shown in Figure 7A. The sensitized Tb^{III} luminescence intensity decreased as the titrating metal ions' concentrations increased, indicating that metal ions compete for a

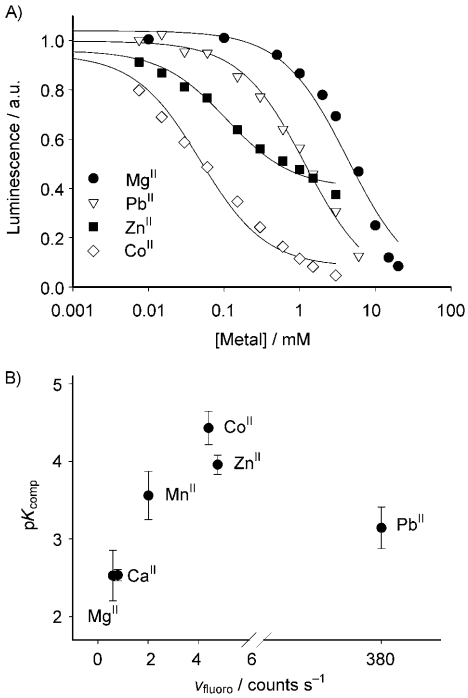

Figure 7. A) Typical sensitized Tb^{III} luminescence curves titrated with divalent metal ions. K_{comp} values were obtained by fitting the curves to nonlinear square equations as described in the Experimental Section. Concentrated metal ions were added to Tb^{III} (15 μM) equilibrated with 17E-DDS (2 μM) in MES (50 mM, pH 6.0), and $NaNO_3$ (50 mM). B) A plot of pK_{comp} versus cleavage reaction rate of v_{fluoro} taken from ref. [11]. v_{fluoro} is the initial fluorescence response rate to the cleavage reaction of a fluorophore-labeled enzyme–substrate complex in the presence of divalent metal ions (500 nM) in HEPES (50 mM, pH 7.5).

binding site or binding sites and drive bound Tb^{III} ion out of the DNAzyme. The Tb^{III} luminescence intensity finally reached background level in the presence of millimolar metal ions except in the case of Zn^{II} ion, which showed residual luminescence. Regrettably, higher Zn^{II} concentrations, which might be required to take all residual Tb^{III} ion out of the DNAzyme, could not be tested because of precipitation problems over 10 mM of Zn^{II}. The competition curves were fit to nonlinear least-squares equations described in the Experimental Section with an assumption that the metals compete for one Tb^{III} binding site in the enzyme–substrate complex. The pK_{comp} values for the metal ions were obtained and shown to be in the order of $Co^{II} > Zn^{II} > Mn^{II} > Pb^{II} > Ca^{II} \approx Mg^{II}$ (Figure 7B), indicating that transition metal ions such as Co^{II} and Zn^{II} bind strongly to the DNAzyme whereas alkaline earth metal ions such as Mg^{II} and Ca^{II} have much weaker affinities. With the exception of Pb^{II}, this order of pK_{comp} closely follows the order of fluorescent sensor selectivity as measured by initial rate of fluorescent

increase for the same group of metal ions.[11] These results suggest that Tb^{III} may bind to the same site as other active metal ions except in the case of Pb^{II}.

Discussion

Similarities in the inhibition and binding of Tb^{III} to the 8–17 DNAzyme and ribozymes: Fully reversible inhibitory effects of Tb^{III} have also been observed in hammerhead[38] and hairpin ribozymes.[40] It has been reported that Tb^{III} binds to the ribozymes tightly, with a $K_{i\,app}$ of 0.2–3.5 μM in the presence of Mg^{II} ion (5–50 mM) for a hammerhead ribozyme,[38] and of 16 μM in the presence of Mg^{II} ion (12 mM) for a hairpin ribozyme.[40] Crystallographic studies showed that Tb^{III} binds to a site adjacent to G5, an unpaired base in the uridine-turn motif of hammerhead ribozyme, where Mg^{II} and Mn^{II} also bind,[38] which plays a significant role for the activity of the ribozyme.[58,59] The similar reversible competitive Tb^{III} inhibition in the 8–17 DNAzyme catalysis suggests that Tb^{III} may bind either to the same or close to the active metal-binding site(s) in the DNAzyme, where Tb^{III} binding can critically affect the DNAzyme activity through, for example, preventing binding of active metal ion(s) or changing the conformation of the DNAzyme. Also, similar numbers of water molecules (two or three) were substituted by functional groups in a hammerhead ribozyme,[39] indicating that Tb^{III} binding modes in these ribozymes and deoxyribozymes are analogous.

Difference in Pb^{II}-dependent catalysis: The Pb^{II} competition with Tb^{III} shown in Figure 7A and B shows relatively low pK_{comp} values for Pb^{II}. One contributing factor is that Pb^{II} ions may have precipitated at high concentrations, resulting in less effective Pb^{II} concentration for the competition binding, and thus that the pK_{comp} value for Pb^{II} may not be as accurate as those for other metal ions. Despite this problem, though, there is still no qualitative correlation between pK_{comp} of Pb^{II} and the apparent k_{obs} of Pb^{II}-dependent activity; while Pb^{II} is the most active and the most selective in DNAzyme activity, it competed with Tb^{III} less effectively than Co^{II}, Zn^{II}, and Mn^{II} as measured by Tb^{III} luminescence spectroscopy. This relatively low pK_{comp} value for Pb^{II} in relation to Tb^{III} is also inconsistent with the findings that much less Pb^{II} (100 μM) was needed fully to rescue the activity inhibited by Tb^{III} (20 μM) than in the case of Zn^{II}, which required ≈2 mM. This observation suggests that Pb^{II} may bind to a different metal-binding site from Tb^{III} and other less active metal ions. Rather than competing directly with Pb^{II} for the same site, Tb^{III} binding may cause indirect local conformational change to disallow a suitable coordination of Pb^{II} in the DNAzyme, resulting in inhibition of the activity. In return, the rescue of Pb^{II}-dependent activity depends mainly on restoration of the appropriate conformation. Inhibition by structural change upon lanthanide binding to an enzyme has been reported previously.[60,61] Activity of the 3′-5′-exonuclease of the Klenow fragment (KF) of *Escherichia coli* DNA polymerase was inhibited by Eu^{III} binding to one of two metal-binding sites, inducing structural changes and thus preventing a critical metal ion from binding to another metal-binding site, the KF exo active site.

This proposal of different binding sites for Pb^{II} than for other metal ions is further supported by recent folding studies of the DNAzyme. Metal-ion-dependent conformational changes and cleavage reactions of the 8–17 DNAzyme have been studied by fluorescence resonance energy transfer (FRET).[31,32] Both bulk and single-molecule FRET studies showed that the DNAzyme folds into a compact structure in the presence of Zn^{II} and Mg^{II}, followed by a cleavage reaction, suggesting that the DNAzyme may require metal-dependent global folding for activation. In the presence of Pb^{II}, however, cleavage occurred without a preceding folding step, suggesting that the DNAzyme does not require global conformational changes for Pb^{II}-dependent activity. Two different reaction pathways in the presence of Pb^{II} and Zn^{II} were proposed on the basis of the FRET studies, accounting for the remarkably fast Pb^{II}-dependent reaction of the DNAzyme.

Conclusion

In summary, Tb^{III} effectively inhibits the Zn^{II}- or Pb^{II}-dependent activity of the 8–17 DNAzyme. Pb^{II} and Zn^{II} compete differently for the Tb^{III} binding. The harder Zn^{II} ion competes competitively, which is indicative of binding to the same, or at least overlapping, Tb^{III} site. For the softer Pb^{II} ion, the evidence suggests the presence of a novel binding site different from that at which Zn^{II} or Tb^{III} bind. The Tb^{III} was found to bind to the unpaired region of the DNAzyme, and the GT wobble pair played a role in Tb^{III} binding. Of the nine possible water molecules in the Tb^{III} coordination sphere, two or three molecules were shown to be replaced by functional groups from the DNAzyme. Further studies including X-ray crystallography and NMR spectroscopy should help in elucidation of the metal-binding sites in the DNAzyme.

Experimental Section

DNAzyme activity assay: HPLC-purified oligonucleotides were purchased from Integrated DNA Technology, Inc. (Coralville, IA). All solutions were made in autoclaved water purified with a Millipore (Milli-Q, Synthesis) system. Concentrated 4-morpholineethanesulfonic acid (MES) buffer was treated with Chelex 100 (Sigma, St. Louis, MO) to remove possible contaminated divalent metal ions in the buffer and was adjusted to pH 6.0 by addition of 99.999% concentrated HCl. The substrate strand (17S) was labeled with γ-^{32}P at the 5′ end and desalted by use of a Sep-pak column (Waters, Milford, MA).

Kinetic studies of the Zn^{II}-dependent cleavage and inhibition reactions in the presence of lanthanide ions were performed in the presence of a 5000-fold excess enzyme strand (17E) under single-turnover conditions. The 17E and the radioisotope-labeled 17S were annealed in MES buffer (50 mM, pH 6.0) by heating at 95 °C for 2 min and cooling to room temperature over 15 minutes. The reaction was initiated by addition of the

2× concentrated Zn^{II} and Tb^{III} mixture to the 2× concentrated DNA solution. The final concentrations were 17E enzyme (5 μM), 17S substrate (1 nM), Zn^{II} (100 μM), and varied concentrations of Tb^{III} as stated. The reaction was quenched at various time points by addition of aliquots (usually 4 μL) of reaction mixture to a stop-buffer containing urea (8 M), EDTA (50 mM), xylene cyanol (0.05%), and bromophenol blue (0.05%). For single-chase experiments, a small volume of concentrated Tb^{III} solution was added to the reaction mixture at certain time points. Double-chase experiments were carried out by addition of Tb^{III} and Zn^{II} in series.

The reaction products and uncleaved substrate were separated by electrophoresis on a denaturing polyacrylamide gel (20%) and analyzed with a Molecular Dynamics Strom 430 PhosphorImager (Amersham Biosciences). The percentage of product was plotted against time and was fitted to pseudo first-order kinetics. The observed rate constant (k_{obs}) was obtained by nonlinear curve fitting with the aid of SigmaPlot 8.0 software on the basis of the equation $y=y_0+a(1-e^{-kt})$, where y is the percentage of product at time t, y_0 is the background cleavage at $t=0$, a is the fraction reacted at $t=\infty$, and k is the observed rate constant (k_{obs}). The extent of inhibition of the DNAzyme cleavage activity by Tb^{III} was studied with different concentrations of Tb^{III}. The data were fitted to the competitive inhibition systems equation: $k=k_{max}[S]/\{K_s(1+[I]/K_{i\,app})+[S]\}$,[62] where k is the observed reaction rate constant (k_{obs}), k_{max} is the maximum observed reaction rate constant in the absence of Tb^{III}, $[S]=[Zn^{II}]$, K_s is [S] required for $^1/_2k_{max}$, and $[I]=[Tb^{III}]$. The apparent inhibition constant, $K_{i\,app}$, was obtained through curve fittings with $K_s(Zn^{II})=997$ μM[52] with the aid of SigmaPlot 8.0.

Terbium luminescence spectroscopy: Sensitized luminescence spectroscopy was performed with a Fluromax-P fluorimeter (HORIBA Jobin Yvon, Inc., NJ). Enzyme and substrate (2 μM each) were annealed in MES (50 mM, pH 6.0) and $NaNO_3$ (50 mM) by heating the mixture at 90 °C for 5 min and then cooling to 4 °C over 3 h. The annealed enzyme–substrate complex was allowed to reach room temperature before each measurement. Sensitized Tb^{III} luminescence titration was carried out by slow addition of concentrated Tb^{III} solution to the DNA complex while the solution was stirred at room temperature. Lifetimes of Tb^{III}–DNA complex adduct in H_2O and in D_2O were obtained with the Fluoromax-P phosphorimeter (HORIBA Jobin Yvon, Inc., NJ). For the lifetime measurements in D_2O, all reagents were dissolved in D_2O (99.9%, Sigma–Aldrich), lyophilized, and then dissolved again in D_2O. All data points include at least three replicates with three different samples.

Metal competition for Tb^{III}-binding site(s): Metal stock solutions of $MgCl_2$, $Ca(NO_3)_2$, $MnCl_2$, $Mn(OAc)_2$, $CoCl_2$, $Co(NO_3)_2$, $ZnCl_2$, and $Pb(OAc)_2$ were prepared from >99.99% pure chemicals. A concentrated Pb^{II} stock solution was prepared in acetic acid (5%) in order to prevent precipitation. Enzyme–substrate complex was prepared as described above in the Tb^{III} luminescence spectroscopy section. Aliquots (1–5 μL) of concentrated metal ion solutions were added to preequilibrated Tb^{III}–DNA adduct while the solution was stirred. Luminescence decrease due to a dilution effect from the metal additions was corrected for by measuring the luminescence changes upon addition of buffer to a sample. The DNA samples were excited at 260 nm, and the corresponding emission at 543 nm was collected. In the case of Pb^{II} titrations, the luminescence was measured at 620 nm to avoid the interference by Pb^{II} luminescence. A 330 nM cut-off filter was used to prevent the second-order diffraction of the grating. Luminescence was fit to an equation, $L_i=L_8+\Delta L\{1-[metal]_i/([metal]_i+K_{comp})\}$, where L_i is the luminescence intensity at a certain concentration i of the metal, L_8 is the luminescence at saturated metal concentration showing minimal luminescence, ΔL is total change of the luminescence over the titration, and K_{comp} is the ratio of the dissociation constants of Tb^{III} to the competitor metal ion, K_d^{Tb}/K_d^{metal}.[38]

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